Occurrence of Pathogenic *Thelohania* (Microsporida: Nosematidae) in the Australian Freshwater Crayfish, *Cherax quadricarinatus* (Decapoda: Parastacidae)

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Thelohania was a major pathogen in experimental *Cherax quadricarinatus* stocks, introduced to North America, infecting late embryos, larvae, juveniles and adults. Spores encysted in heart, limb and abdominal muscles, small numbers of spores were also detected in ovarian and neural tissues. The vigorous but incomplete host response included melanization in most tissues and concentration of spores in the tips of gill filaments; there was no apparent immune reaction in neural tissues.

Evidence for direct transmission from parent to embryo is presented. Trials with asymptomatic groups indicated that thermal stress (3-6h exposure to temperatures above 32°C) would induce thelohaniasis symptoms within hours. Regression of these symptoms was also observed when some infected individuals were maintained at lowered temperatures (27-31°C). *C. quadricarinatus* populations free of *Thelohania* survived protracted exposure to temperatures of 35-37°C.

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INTRODUCTION

After the plague fungus (*Aphanomyces astaci*), microsporidial protozoans of the genus *Thelohania* are responsible for the most important disease problems in freshwater crayfish throughout the world (Alderman and Polglase, 1988); *Thelohania* species are known from North America (Sprague, 1950), the United Kingdom (Cossins, 1973), Europe and Russia (Voronin, 1971; Vey and Vago, 1973) and Australasia (Quilter, 1976; Mills, 1983). Levels of incidence reported in wild populations have ranged from 0.3-38% in Australia (Herbert, 1988; O'Donoghue *et al.*, 1990) and 10-30% in western Europe (Alderman and Polglase, 1988).

Endoparasitic microsporidians infect a range of crayfish species, including some of economic importance; however, their taxonomy is still confused and life cycles are poorly known (Langdon, 1990) — current knowledge is summarized below.

Identification and Classification

Examination of scrapings or squashes of infected tissues, using a compound light microscope, is required to ascertain if spores and pansporoblasts are present (Merrick and Lambert, 1991: 125). Stains such as Giemsa, haematoxylin and eosin, will highlight spore recognition features (Herbert, 1988). Individual mature *Thelohania* spores are pyriform (3.00-3.69 μm length: 2.00-2.35 μm width) refractile in transmitted light and phase bright; 6-7 polar filament windings and 3 layers in the spore wall can be observed with electron microscopy (O'Donoghue *et al.*, 1990). Each sporocyst contains a maximum of 8

spores, but dimensions of the spore masses (xenomas) which then develop vary considerably — up to 2mm in length and 7-80μm in width; the xenomal wall is reported to be smooth — without invaginations or septae (Herbert, 1988; O'Donoghue *et al.*, 1990).

Although the validity of the genus has not been questioned the familial designation, and numbers of species recognized within the genus, have been subjects of controversy. Usually assigned to the family Thelohaniidae, *Thelohania* was referred to Nosematidae in recent revisionary studies (Hazard and Oldacre, 1975); but it is below the generic level that most taxonomic confusion exists.

Langdon (1990) noted that many species have been assigned to *Thelohania* and infect vertebrates as well as other invertebrate groups. From the *Thelohania* material recorded from crayfish several distinct species have been described; but it is still not clear if the *Thelohania* group parasitizing crayfish comprises a small number of widespread species, each infecting many populations of various crayfish species, or a larger number of isolated parasites adapted to particular hosts (Sprague and Couch, 1971; Kelly, 1979).

Life Cycle

In the absence of detailed information *Thelohania* probably follows the general microsporidian cycle. This general pattern involves initial ingestion of spores by the crayfish; once in the intestine the spore extrudes a polar filament which penetrates the gut wall. The spore cytoplasm is believed to be liberated through the filament and having penetrated the intestinal epithelium it enters an intestinal muscle fibre. Asexual division within the muscle produces schizonts, each of which then divides repeatedly to produce a pansporoblast containing 8 sporoblasts within a membrane. Spores may be liberated singly or in the pansporoblast — more muscle cells are subsequently infected by individual spores, so the infection spreads. Although primarily restricted to muscle, spores have been found in the nervous system, the connective tissue surrounding the gut, ovary, developing eggs and in the haemocoel (Johnson, 1977; Alderman and Polglase, 1988).

Generally crayfish show little reaction to this intracellular parasite, aside from an inflammatory response associated with muscle cell rupture; most parasites apparently remain as pansporoblasts within the converted muscle cells. Death is considered inevitable, resulting from increasing destruction of muscle — especially buccal and heart muscle; however, survival times vary widely. Infected individuals may take 5 or 6 months to show clinical symptoms and then take a further 12 months to die (Alderman and Polglase, 1988).

Thelohania only appears to be transmitted by the ingestion of spores or infected tissues containing them (Alderman and Polglase, 1988). Fish and invertebrate secondary hosts or intermediate stages have been suggested (Chartier and Chaisemartin, 1982; Herbert, 1988; Langdon, 1990) but no evidence supporting these ideas has been presented; the possibility of transovarian transmission has also been raised, but observations to date have not confirmed it (Alderman and Polglase, 1988).

There is no known treatment for thelohaniasis in crayfish and the only control measure is to regularly monitor the population — removing any specimens showing symptoms. Factors suggested as influencing outbreaks of this disease include crayfish population density, high temperatures and low pH (Alderman and Polglase, 1988; Langdon, 1990).

The objectives of studies reported here, on the northern Australian endemic *Cherax quadricarinatus,* were: to document initial symptoms and host immune reactions to *Thelohania* infection; to investigate the evidence for possible alternative modes of transmission; and to observe host-parasite interaction under selected thermal conditions.

Materials and Methods

Experimental stocks were derived from a consignment of 20,000 *Cherax quadricarinatus* juveniles (approx. 10mm TL) obtained from commercial suppliers in Queensland and held in isolation in Alabama. These imported stocks were housed in two newly constructed 0.125 ha ponds, filled and topped up with bore water, and monitored for eight months. A sample of 1400 adults (>30g) was removed from the ponds to hatcheries and the trials reported were done on the progeny produced. The hatcheries were monitored, twice daily, for a period of eight months. Any mortality or disease was investigated by the author with the assistance of the Disease and Parasite Laboratory, Department of Fisheries, Auburn University.

All tissue samples were examined with a compound light microscope and *Thelohania* spores identified from fresh and stained smears. No classification below the generic level was attempted. Reference samples of diseased tissue were retained in preservative, but other diseased remains were disposed of by incineration.

Observations of reactions to initial infection and the occurrence of spores are listed and results of laboratory trials, in which infected individuals were subjected to high temperatures, are summarized in Table 1.

Results

Identification, Initial Infection

Microscopic observation of mature *Thelohania* spores from abdominal muscle showed that they were uniform in size (3.0μm x 2.0 μm) and lightly basophilic, whereas sporonts and sporoblasts were eosinophilic. Examination of frozen tail muscle revealed only spores; no intact pansporoblasts, as observed in fresh tail muscle and other tissues.

During the initial stages of infection *C. quadricarinatus* appeared normal and the first observable external symptom was the development of white or grey streaks in the anterior ventral abdominal musculature. This discolouration corresponded to a concentration of spores and the formation of xenomas.

The abdominal infection (muscle destruction) then spread posteriorly; however, examinations of a number of adults revealed spores, pansporoblasts and xenomas in muscles of the heart, chelipeds and pereiopods. Small numbers of spores were also present in connective layers surrounding the ovaries and neural tissues. Immune responses observed included melanization in most tissues and the concentration of spores in distal portions of gill filaments. This latter reaction of aggregation and melanization in gill filaments was conspicuous in larvae.

As the trials reported below demonstrate, *Thelohania* also infects embryos, larvae and juveniles of *C. quadricarinatus*.

Environmental Stress

Newly fertilized eggs when removed from healthy females and incubated in isolation, did not survive to hatching at any temperature; despite all precautions total mortality resulted from fungal infections. By contrast, a large number of eggs (totalling approximately 30,000) removed at blastula, early gastrula or later stages had hatching rates of 30%-98% if maintained at 18-31°C. Furthermore, up to 60% of late stage embryos (incubated in isolation) survived prolonged exposure (>3h) to temperatures of 32-36°C. Batches of larvae (from eggs incubated in isolation) had survival rates of 60%-100% after 3-6h exposure to temperatures of 32-36°C, but larvae maintained below 31°C had less than 5% mortality. Likewise juveniles exposed to high temperatures had survival rates of up to 40%, whereas 80-95% of those below 31°C survived (Table 1).

Most of the mortality associated with the artificial incubation trials involving late

TABLE 1

Summary of samples, treatments and results of thermal stress trials during the period October 1989 to June 1990

Stage	Location	Total number in sample (sub-samples)	Treatment	Observations	Duration
EGGS Spawning-nauplius Day 0-4	On females	2,000 (20)	3-6 hrs @ 32°-36°C	Mortality* was 100% in both attached and isolated eggs. Chorion became discoloured (whitish); fungi developed on chorion prior to death.	To hatch 11-43 Days
	Isolated†				
	On females	7,000 (70)	22°-31°C	Mortality 5-10%	
	Isolated			Mortality 100%	
LATE STAGE Post nauplius — eyes pigmented Day 5 to 14 EMBRYOS	On females	2,000 (20)	3-6 hrs @ 32°-36°C	Mortality 60-100% when all females died. Muscles of abdomen, pereiopods, chelipeds and thorax became clouded then completely white as movement decreased. Bundles of pansporoblasts in early stages and extensive pansporoblasts in later stages.	
	Isolated			Mortality 40-100% in petri dishes.	To hatch 9-36 Days
	On females	7,000 (70)	18°-31°C	Average mortality 2-10% (some female cannibalism).#	
	Isolated			Mortality 2-70% in petri dishes.	
LARVAE Stage 1 & Stage 2 Day 15-79	On females	3,000 (30)	3-6 hrs @ 32°=36°C	Mortality up to 100%. Muscles of abdomen, pereiopods, chelipeds and thorax became clouded, then completely white as movement and tail flock response decreased. Bundles of pansporoblasts in early stages and extensive pansporoblasts in later stages as well as free spores in circulatory system.	
	Isolated			Mortality 0-40% in petri dishes.	To release 14-42 Days
	On females isolated	30,000 (30)	22°-31°C	Mortality 0-5% in both attached and isolated larvae.	
JUVENILES Release to 30cm T.L. (Maturation)		2,000 (4)	3-6 hrs @ 32°-37°C	Mortality 60-100%. Symptoms as for larvae.	
		30,000 (30)	22°-31°C	Mortality 5-20%.	60 Days
ADULTS	Berried Females	3	33°-38°C	Mortality 100%. These females held in isolation developed symptoms within 3-96 hrs of temperature increase, all died 3-144 hrs after symptoms first observed; free spores, pansporoblasts.	
		300	20°-31°C	Mortality 0%.	8 Days

* Mortality values relate to different time spans: in egg/embryo stages it is taken to hatching; in larvae it is calculated as the loss up to release (yolk absorption).
† Isolated = detached from parent, maintained in petri dish.
~ Wide variation in duration due to different temperatures.
#Some subsamples showed 100% mortality due to female cannibalism.

embryos at temperatures >33°C can be attributed to *Thelohania*, as spores occurred in muscle tissue. Samples of larvae which had been exposed to the general population and subjected to high temperatures (33°-36°C), developed a very high incidence of thelohaniasis within 6-72 h.

Clinical symptoms of thelohaniasis were often observed following rough handling or moulting. Up to 10% of larval, juvenile and adult *C. quadricarinatus* presented symptoms 3-8 days after ecdysis.

It was also noted that in some juveniles subjected to thermal stress the initial infection symptoms began to regress when the animals were returned to temperatures below 31°C. The anterior abdominal cysts began to clear — from the original infection site first, then posteriorly.

Discussion

Thelohaniasis is the most important disease of crayfish in Australia; it has been reported in South Australia, Victoria, New South Wales and Queensland (O'Donoghue *et al.* 1990; Merrick and Lambert, 1991: 121) and at least three *Cherax* species may be infected (Mills, 1983; Herbert, 1987; Semple, 1993, unpublished data). It is also acknowledged that previous reports of infection levels — largely based on individuals exhibiting clinical symptoms — are underestimates.

Identification

As mentioned previously the number of *Thelohania* species parasitizing crayfish is unknown. Little variation in mature *Thelohania* spores has been observed in Australia (Herbert, 1988; O'Donoghue *et al.*, 1990); however, some variation in spore size from different hosts has been reported between continents (Cossins and Bowler, 1974; Quilter, 1976; Herbert, 1988). The determinations are based on fresh squashes and the presence of separate spores only in frozen material is considered due to lysis of the pansporoblast walls; this phenomenon of lysis as a result of the freezing process has also been reported in prawns (Owens and Glazebrook, 1988).

Although the specific identity of the *Thelohania* studied was not determined, the evidence suggests that it was an Australian species imported with the experimental stocks. Reasons for drawing this conclusion include: no observation of any mortality in experimental stocks with *Thelohania* symptoms prior to transfer to hatchery; the new, isolated ponds supplied with bore water — in an area where thelohaniasis has not been reported; the thorough preparation of hatchery facilities where *Thelohania* had not previously been recorded. The probability of contamination from an external source is very low; furthermore, the direct transmission mode (transfer of spores from adults to developing eggs), as demonstrated by these studies, indicates a mechanism to explain how the disease could have been present although undetected.

Initial Infection, Transmission

Immune reactions to *Thelohania* were observed in all stages of *C. quadricarinatus* from larvae to adults; however, host response was incomplete. The observation of Herbert (1988) that there was no apparent response to the small number of spores associated with nerve tissue was confirmed.

The overall distribution of spores in adult tissues was also as reported by Herbert (1988) and is consistent with transmission by means of ingestion and invasion from the gut. Whilst the studies reported here do not corroborate this indirect transfer, characteristic of microsporidians, they do demonstrate a second direct transmission mode in *Cherax quadricarinatus*. This is the first time that *Thelohania* has been reported from developmental stages in an Australian species.

Another *Thelohania* species is known to be transferred directly in vertebrate host eggs (Post, 1987: 173); furthermore, Voronin (1971) reported *Thelohania* spores in embryos of an astacid crayfish. Microscopic examination did not reveal spores in C. *quadricarinatus* oocytes, but they were present in the ovarian wall. Furthermore, it should be emphasized that detection of occasional spores in oocytes would be difficult because of the dense, granular nature of oocyte contents. Although spores were not actually observed in *C. quadricarinatus* ova, their occurrence in ovarian tissues and embryos (4 days old) indicates that they were almost certainly present.

The only alternative explanation is that free mature spores infected eggs rapidly by directly entering ova at release, during fertilization or soon after. It is clear that *Thelohania* does not need an intermediate host for positive activation in *Cherax quadricarinatus*, however, the trials reported do not preclude the possibility that, under some conditions, intermediate vectors may be involved in transmission.

Previous arguments for the involvement of intermediate hosts have centred around the need for priming of the spore; however, the present research indicates the possibility that spores could be primed by a physicochemical environmental factor, such as temperature or pH, without the need to enter another vector. The occurrence of *Thelohania* spores in other benthic invertebrates, such as simuliids (Chartier and Chaisemartin, 1982), does not mean that they are intermediates.

Environmental Stress

The detection of spores in fresh tissue squashes eliminates the possibility that the macroscopic clinical symptoms were due to lactic acidosis induced by thermal stress.

Langdon (1990), in general comments about microsporidians, noted that: their incidence in crayfishes decreased with increased latitude; incidence increased with crowding and high temperatures. The implication was the incidence may be related to stressful conditions. These studies have confirmed this suggestion; however, it is not clear whether initiation of parasite activity is due to priming of spores, lowering of host immunity or both.

The lack of response in previous infection trials with *C. quadricarinatus* (Herbert, 1988) may be, at least partly, due to the relatively low temperatures (18-24°C) at which experimental stocks were held.

Other observations which support the hypothesis of environmental stress influencing *Thelohania* activity include: the 10% incidence reported in *C. quadricarinatus* up to 8 days after ecdysis; infections, in small numbers of *C. destructor*, which developed after short exposure to lowered dissolved oxygen levels (Semple, 1993, unpublished data).

The observation of regression of symptoms in animals returned to lowered temperatures is significant in two ways. It is the first indication that this infection is reversible and secondly may assist in the development of control measures.

Management

The present studies have not fully elucidated the transmission of *Thelohania*, but the results have several broad implications for crayfish conservation and culture in Australia. Firstly, of the Australian species carrying *Thelohania, Cherax quadricarinatus* and *C. destructor* are extensively cultured and have been widely translocated. The possible impact of disease outbreaks in cultured stocks on other indigenous parastacids, many of which have restricted distributions, is unknown (Merrick, 1993: 90-92) but should be investigated urgently.

Secondly, the role of environmental stress should be considered when decisions are taken on culture at the margin of, or outside, a species range. In these marginal areas limiting environmental conditions (for that species) are likely to be encountered periodically; this pathogen could be present (but asymptomatic) for many months and then be rapidly activated by a short period of extreme conditions.

Thirdly, the direct transmission demonstrated means that screening or quarantine procedures should include examinations of nerve tissues, eggs and larvae as well as muscle; short duration thermal testing may also be useful.

Finally, a key observation emerging from these studies is that this infection is not necessarily irreversible. The immune reactions observed from larva to adult may, in combination with manipulation of selected environmental factors, form the basis of a control strategy.

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